

Relict Distribution in *Pedicularis spicata* Pall. (Orobanchaceae): a New Locality in Central Honshu, Japan

NORIYUKI FUJII^{1,*}, MIHOKO TERAMOTO¹, RICHARD H. REE², NOBUKAZU SHIRAI³,
CHIKA SUYAMA⁴, KUNIHICO UEDA⁵ AND HIDEKI TAKAHASHI⁶

¹Graduate School of Science and Technology, Kumamoto University, 2-39-1
Kurokami, Chuo-ku, Kumamoto, Kumamoto 860-8555, Japan.

*nfujii@kumamoto-u.ac.jp (author for correspondence);

²Department of Botany, Field Museum of Natural History, 1400 S Lake Shore Drive,
Chicago, IL 60605, USA; ³Tawara-machi, Kanazawa, Ishikawa 920-1108, Japan;

⁴Faculty of Education, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan;

⁵Graduate School of Natural Science and Technology, Kanazawa University,
Kakuma, Kanazawa, Ishikawa 920-1192, Japan; ⁶The Hokkaido University
Museum, Kita 10, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0811, Japan

We report a new locality for *Pedicularis spicata* Pall. (Orobanchaceae) in the Hakusan Mountains, Honshu, Japan. Although the populations of Hakusan Mountains have been described as *P. verticillata*, we showed that they are *P. spicata*. *Pedicularis spicata* is identical with the Hakusan populations in *trnK* sequences of chloroplast DNA (cpDNA), whereas internal transcribed spacer (ITS) phylogeny unites a clade of *P. spicata* and the Hakusan populations at 94% bootstrap support. Morphological analyses further suggest that the population at Hakusan Mountains should be included in *P. spicata* rather than *P. verticillata*. *Pedicularis spicata* occurs mainly in northeastern Asia. This study shows a disjunct pattern for *P. spicata* between the Asian mainland and Honshu, Japan.

Key words: central Honshu, disjunct distribution, Hakusan Mountains, new locality,
Pedicularis spicata, *Pedicularis verticillata*

The flora of the Japanese archipelago has been relatively well investigated with the results summarized in the floristic literature (Ohwi 1965, Iwatsuki *et al.* 1993, 1995a, 1995b, 1999, 2001, 2006). Many new taxa and geographic occurrence records, however, continue to be reported (Setoguchi & Maeda 2010, Oda & Nagamasu 2011, Kadota 2012, Suetsugu *et al.* 2012, Sugawara 2012, Fujii *et al.* 2013). Such findings are important, because they can prompt the reexamination of previous taxonomic assignments and delimitation of taxonomic boundaries. Further-

more, improvements to the quality and accuracy of geographic distributions are important for understanding the biogeographic origins of the Japanese flora (Murata 2000).

Pedicularis verticillata L. (Orobanchaceae) is an annual alpine herb that has been classified into ser. *Verticillatae* Maxim. of sect. *Sigmantha* H. L. Li (Yamazaki 1993). This species is widely distributed across the high latitudes of the northern hemisphere and thus is a circumpolar arctic–alpine plant. In Japan, it shows a restricted distribution pattern and has been recorded in the Taisetsu

Mountains (Mtns.) in Hokkaido and on several high mountains in central Honshu (Mt. Shirouma, Mt. Shibutsu, Yatsugadake Mtns., Akaishi Mtns., and others) (Yamazaki 1993, Shimizu 1997). The occurrence of *Pedicularis verticillata* on the Hakusan Mtns., Ishikawa Prefecture, central Honshu, Japan, has also been reported in the literature (Hashimoto & Satomi 1969, Satomi 1983). In a recent detailed inventory of the mountains, *P. verticillata* was reported along with the description of a voucher specimen (Hakusan Nature Conservation Center 1995). The populations occur in the subalpine–alpine zone at an elevation of 2,000 m or more. It was observed, however, that the morphological characteristics of the plants (size and inflorescence traits) slightly differed from *P. verticillata* on other mountains in Japan (N. Shirai & T. Shimizu unpublished). Fujii *et al.* (1996) examined variation in chloroplast DNA (cpDNA) among the populations of *P. verticillata* in Japan, including those on Hakusan Mtns., and observed a large sequence differentiation between the Hakusan accessions and those from other mountains. Based on the results, it was inferred that the Hakusan populations might not be *P. verticillata*.

To clarify the identity of the Hakusan populations, we reexamined them using the taxonomic literature and molecular phylogenetic analysis. The examination suggested that the Hakusan populations were *Pedicularis spicata* Pall (Fig. 1), a species, like *P. verticillata*, classified in ser. *Verticillatae* of sect. *Sigmantha* (Yamazaki 1993). *Pedicularis spicata* is confined to eastern Asia, ranging from Hokkaido to northern Korea, north-eastern China, northern China and Mongolia to eastern Siberia (Ivanina 1991, Yamazaki 1993, Yang *et al.* 1998, Grubov 2001) (Fig. 2). On the Japanese Islands, *P. spicata* has been reported only in the Tokachi district of Hokkaido. It was suggested that it might have been introduced from northeastern China (Yokoyama 1951, Takita 2001). Until now, *P. spicata* has not been reported on the Honshu Island, Japan.

To determine the identity of the Hakusan plants, we performed molecular phylogenetic analyses and morphological comparisons with

closely related species of *Pedicularis* ser. *Verticillatae* (*P. spicata*, *P. verticillata*, and *P. refracta* (Maxim.) Maxim.) in Japan.

Materials and Methods

Materials

For molecular phylogenetic analyses, DNA sequences of the nuclear internal transcribed spacer (ITS) region and the plastid *trnK* 5'-terminal region (including a part of *matK*) were determined for three accessions from the Hakusan populations, one accession of *Pedicularis spicata*, five accessions of *P. verticillata*, and three accessions of *P. refracta*, excluding the accessions from Mt. Kita and Mt. Tolbachik (Table 1). All sequence data were deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB754816–AB754828. The new sequence data, including the ITS and *trnK* sequences, were assembled into alignments of eight species (15 accessions) previously published by Ree (2005) and Yang & Wang (2007). Of these species, *P. lineata* Franch. ex Maxim. and *P. szetschuanica* Maxim. form a strongly supported clade with *P. verticillata*, whereas *P. anas* Maxim., *P. megalantha* D. Don and *P. longiflora* Rudolph represent outgroups (Ree 2005). Voucher specimens newly used in the present study were deposited in the herbarium of the Faculty of Science, Kumamoto University (KUMA); Makino Herbarium of Tokyo Metropolitan University (MAK); and the herbarium of the Arnold Arboretum, Harvard University (A).

For morphological analyses, we examined herbarium specimens at the University of Tokyo (TI), Kanazawa University (KANA), and the Botanical Garden of Hokkaido University (SAPT). In addition, we used dried specimens prepared from our own field collections of the Hakusan populations and of *Pedicularis refracta* (from Takamori-cho, Kumamoto Pref., Kyushu, Japan). In total, we examined 20 specimens of the Hakusan populations, 127 of *P. verticillata*, 44 of *P. spicata*, and 23 of *P. refracta*. Voucher specimens for our collections were deposited in KUMA.

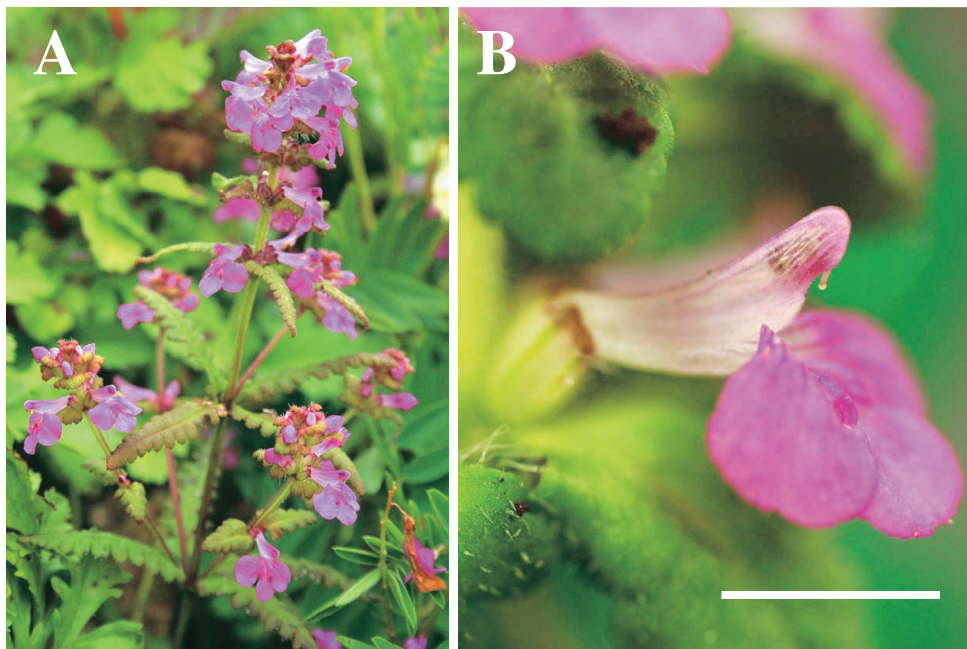


FIG. 1. *Pedicularis spicata* in the Hakusan Mtns., Honshu, Japan. A. Habit (Mt. Shaka–Mt. Nanakura, August 14, 2006). B. Close-up of flower (Mt. Shaka–Mt. Nanakura, August 5, 2010). Scale: 5 mm.

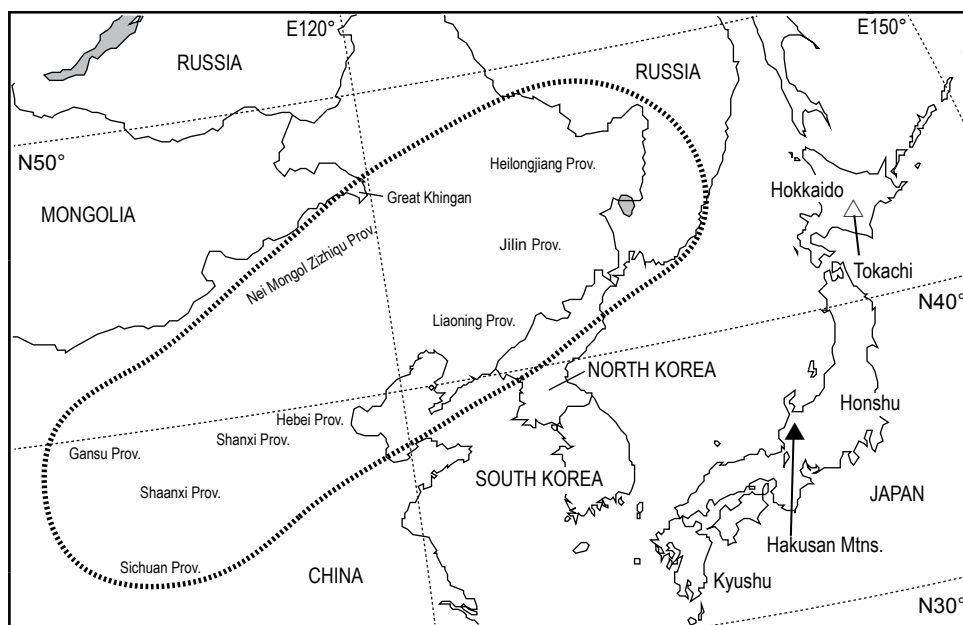


FIG. 2. Distribution map of *Pedicularis spicata*. The distribution range on the Asian continent roughly is shown on the basis of Grubov (2001), Ivanina (1991), Yamazaki (1993), and Yang *et al.* (1998). In Japan, this species were reported only in the Tokachi region, Hokkaido; the possibility of its introduced origin from northeastern China is pointed out (see text).

TABLE 1. Materials used for phylogenetic analyses.

Species, sample number and location		Collector, voucher or reference	Accession Nos.	
			ITS	<i>trnK</i>
Hakusan populations				
1	Mt. Shaka—Mt. Nanakura, Ishikawa Pref., Honshu, Japan	<i>N. Fujii F12115</i> (KUMA)	AB754816	AB754825
2	Mt. Shaka—Mt. Nanakura, Ishikawa Pref., Honshu, Japan	<i>N. Fujii, DNA sample No. F1165</i> (no voucher)	AB754817	AB754825
3	Mt. Sannomine, Gifu Pref., Honshu, Japan	<i>T. Shimizu 176148</i> (KANA)	AB754816	AB754825
<i>Pedicularis spicata</i> Pall. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)				
4	Primorskij region, Russia.	<i>K. Ueda & C. Suyama 1575a</i> (KANA)	AB754818	AB754825
5	Mentougao, W. Beijing, China	<i>YANG0401, Yang & Wang</i> (2007)	AY881148	---
<i>P. verticillata</i> L. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)				
6	Mt. Kita, Nagano Pref., Honshu, Japan	<i>N. Fujii 198987</i> (KANA)	AB754819	---
7	Mt. Yatsugadake, Nagano Pref., Honshu, Japan	<i>N. Fujii F00655</i> (MAK)	AB754819	AB754826
8	Mt. Shibutsu, Gunma Pref., Honshu, Japan	<i>H. Yoshii F00092</i> (MAK)	AB754820	AB754826
9	Taisetsu Mtns., Hokkaido, Japan	<i>NF151, Ree</i> (2005)	AY949698	AY949762
10	Mt. Tolbachik, Kamchatka Peninsula, Russia	<i>S. Okitsu 195143</i> (KANA)	AB754821	---
11	Jiuzhi Xian, Qinghai Prov., China	<i>Boufford et al. 39306</i> (A)	AB754822	AB754827
12	Switzerland	<i>RN, July 15, 1999, Ree</i> (2005)	AY949706	AY949769
<i>P. refracta</i> (Maxim.) Maxim. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)				
13	Kokonoe, Oita Pref. Kyushu, Japan	<i>M. Teramoto & N. Fujii T018</i> (KUMA)	AB754823	AB754828
14	Takamori, Aso, Kumamoto Pref., Kyushu, Japan	<i>M. Teramoto & N. Fujii T2</i> (KUMA)	AB754823	AB754828
15	Hitooyoshi, Kumamoto Pref., Kyushu, Japan	<i>M. Teramoto H1</i> (KUMA)	AB754824	AB754828
<i>P. lineata</i> Franch. ex Maxim. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)				
16	Maerkang Xian, Sichuan Prov., China	DEB 27942, Ree (2005)	AY949664	AY949734
<i>P. szetschuanica</i> Maxim. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)				
17	Sichuan, China	DEB 27708, Ree (2005)	AY949690	AY949754
Outgroups				
<i>P. anas</i> Maxim. (ser. <i>Verticillatae</i> , sect. <i>Sigmantha</i>)			AY949700	AY949764
<i>P. megalantha</i> D. Don (ser. <i>Megalanthae</i> , sect. <i>Saccochilus</i>)			AY949656	AY949727
<i>P. longiflora</i> Rudolph (ser. <i>Longiflorae</i> , sect. <i>Schizocalyx</i>)			AY949645	AY949721

Molecular phylogeny

Leaves of the sample plants were dried and preserved in silica gel. After powdering the leaves in a mixer (Multi Bead Shocker MB455U, Yasui Kikai Corp, Osaka, Japan), total genomic DNA was extracted from 0.01 g of the powder using a slightly modified version of the cetyltri-

methyl ammonium bromide (CTAB) method described by Doyle & Doyle (1987). Polymerase chain reaction (PCR) was performed for the ITS and the *trnK* regions using the following primers [ITS: ITS5 and ITS4 (White *et al.* 1990), *trnK*: trnK11 and matK510R (Young *et al.* 1999)]. The PCR reaction mixtures contained 50–100 ng of

template DNA, 6 μ L of $10 \times$ PCR buffer, 0.2 mM of each deoxyribonucleotide, 2.0 mM of $MgCl_2$, 0.4 μ M of each of the primer pairs, and 1.0 U of ExTaq DNA polymerase (Takara Bio Inc., Tokyo) in a total volume of 60 μ L. The PCR program ran for 3 min at 94°C for initial denaturation, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min and extension at 72°C for 2 min. The reactions were then extended by 7 min at 72°C. PCR products for direct sequencing were excised from 1.5% agarose gels and purified using the GENECLEAN III Kit (Qbiogene, Inc., Carlsbad, CA). Sequencing reactions were performed using the CEQ dye terminator cycle sequencing Quick Start kit (Beckman Coulter, Inc., Fullerton, CA) according to the manufacturer's recommendations. The following internal sequencing primers were used to read the whole regions: ITS: ITS3 and ITS4 (White *et al.* 1990), *trnK*: matK-53F (Young *et al.* 1999), *trnK*600R (newly designed primer, 5'-AACTCG-GAGACAGGTAACTT-3') and matK840R (newly designed primer, 5'-TGTCATAACCTG-GATTTTCC-3'). The sequencing reaction products were purified, concentrated by EtOH precipitation, and then subjected to the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.).

The resulting nucleotide sequences were edited using ChromasPro ver. 1.42 (www.technelysium.com.au/ChromasPro.html) and aligned using MEGA ver. 5.05 (Tamura *et al.* 2011). Phylogenetic analysis was conducted using the neighbor-joining (NJ) method using MEGA ver. 5.05

and the maximum parsimony (MP) method using PAUP* ver. 4.0b10 (Swofford 2003). In NJ analysis, genetic distances were calculated for both regions (ITS and *trnK*) using the Kimura two parameter model (Kimura 1980). Insertions/deletions (indel) and missing data were treated as complete deletions. Clade support was estimated using bootstrap analysis (Felsenstein 1985) based on 10,000 replicates. In the MP analysis, the trees were solely constructed on the basis of substitution data and using the branch and bound search option with MulTrees on. The addition sequence option was set at 'furthest'. Clade support was estimated using bootstrap analysis based on 1,000 replicates in PAUP* ver. 4.0b10. In the analysis, the heuristic search option was used, with the TBR branch swapping option and the 'simple' stepwise addition option.

Morphological analyses

Seven characteristics were measured on dried specimens to determine the taxonomic affiliation of the Hakusan populations: (a) angle of apex of the galea (upper lip of the corolla), (b) corolla length (excluding the calyx tube), (c) leaf blade length, (d) width between rachis and longest pinna, (e) width between rachis and sinus of the pinnae, (f) number of pinnae, and (g) plant length (Fig. 3). In addition, we measured the largest flowers, leaves, and plants. The measurements, excluding plant length, were taken using a portable digital scope (Handy Scope 130s, Science-Eye, Saitama) with DinoCapture 2.0 ver. 1.3.5

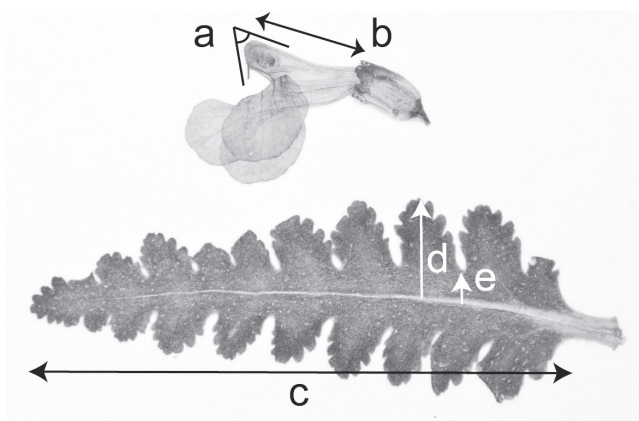
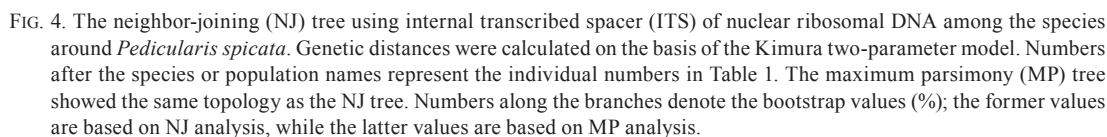


FIG. 3. Floral and leaf blade characteristics measured for analysis of variance. a: angle of the apex of galea, b: corolla length excluding calyx tube, c: leaf blade length, d: width between rachis and longest pinna, e: width between rachis and sinus of the pinna. In addition to above-mentioned characteristics, we measured number of pinnae (f), plant length (g), and calculated the ratios of the characteristics c/d (h) and d/e (i). The flower and leaf in the figure are from plants of the Hakusan populations (No. 1, Table 1).



Individual ITS sequences ranged in length from 571 to 575 bp. The aligned ITS data set was 578 bp in length (including outgroups). The alignment included the 5.8S ribosomal RNA coding region (164 bp). Pairwise distances between species/populations as determined using MEGA from the uncorrected *p*-distance matrix ranged

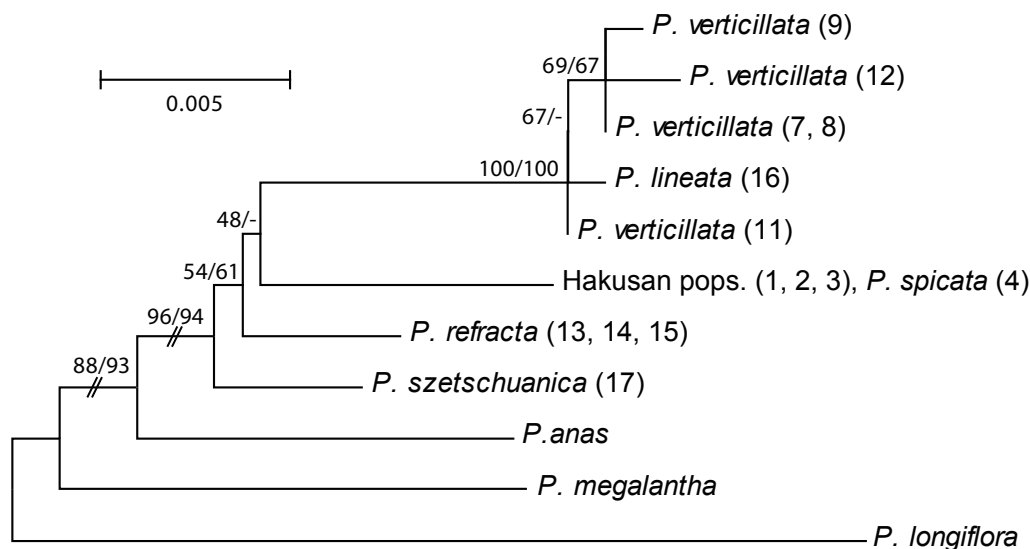


FIG. 5. The neighbor-joining (NJ) tree using the *trnK* region of chloroplast DNA among the species around *Pedicularis spicata*. Genetic distances were calculated on the basis of the Kimura two-parameter model. Numbers after the species or populations names represent individual numbers in Table 1. The maximum parsimony (MP) tree showed essentially the same topology as the NJ tree. Numbers along the branches denote the bootstrap values (%); the former values are based on NJ analysis, while the latter values are based on MP analysis.

from 0.2% between accessions of *Pedicularis verticillata* (No. 6, 7 vs. No. 8) to 5.9% between accessions of *P. verticillata* (Nos. 6, 7, and 12) and *P. refracta* (Nos. 13 and 14) (Table 1).

Sequences of the *trnK* region used in the present study ranged from 1026 to 1036 bp, and the aligned data set was 1044 bp in length. The alignment included 377 bp of the *matK* coding region. The DNA sequences of the Hakusan populations were identical with *Pedicularis spicata* in this region. Intraspecific variation in *P. refracta* was not detected. Values of the pairwise *p*-distance ranged from 0.1% between accessions of *P. verticillata* (Nos. 7 and 8 vs. Nos. 9 and 11) and those of *P. verticillata* and *P. lineata* (No. 11 vs. No. 16) to 1.9% between those of *P. verticillata* and *P. refracta* (Nos. 1–4 vs. No. 12).

Phylogenetic analyses

The NJ tree for ITS exhibited the following phylogenetic relationships (Fig. 4): First, the ingroup accessions, including the Hakusan populations, *Pedicularis spicata*, *P. refracta*, *P. verticillata*, *P. lineata*, and *P. szetschuanica*, formed a

clade with a 99% bootstrap value. Second, two major clades were identified within the ingroup accessions. One clade comprised the Hakusan populations, *P. spicata*, and *P. refracta* with a 97% bootstrap value. Within the clade, the Hakusan accessions and *P. spicata* were monophyletic with a bootstrap value of 94%. The other clade included *P. verticillata*, *P. lineata*, and *P. szetschuanica*, supported by a bootstrap value of 44%. Within this clade, the accessions of *P. verticillata* were monophyletic with 99% bootstrap support, and those of *P. lineata* and *P. szetschuanica* formed a clade with a bootstrap support of 100%. In the MP analysis for the ITS region, three MP trees, requiring 185 steps, were obtained. The consistency index, including uninformative characters (CI), was 0.8486 and the retention index (RI) was 0.8627. The strict consensus tree of the three trees had the same topology as the NJ tree (Fig. 4).

The NJ tree constructed using the *trnK* region of cpDNA is depicted in Fig. 5. The ingroup accessions constituted a single clade with 96% bootstrap support. Bootstrap support for branch-

TABLE 2. Analysis of variance of morphological characteristics between Hakusan populations, *Pedicularis spicata*, *P. verticillata*, and *P. refracta*.

	Hakusan population	<i>P. spicata</i>	<i>P. verticillata</i>	<i>P. refracta</i>
a) angle of apex of the galea (°)	52.9±12.5 C ¹ (N=20)	50.8±10.6 C (N=27)	83.9±11.3 A (N=95)	66.9±10.7 B (N=23)
b) corolla length (mm)	8.9±0.8 C (N=20)	10.1±1.8 C (N=26)	11.0±1.3 B (N=92)	15.1±2.3 A (N=23)
c) leaf blade length (mm)	40.4±10.8 A (N=20)	43.2±17.5 A (N=34)	20.0±7.7 B (N=66)	34.6±7.6 A (N=16)
d) width between rachis and longest pinnae (mm)	6.8±2.2 AB (N=20)	5.4±1.8 BC (N=34)	4.4±1.8 C (N=66)	0.8±0.4 C (N=66)
e) width between rachis and sinus of the pinnae (mm)	2.5±0.9 A (N=20)	1.8±0.7 B (N=34)	0.8±0.4 C (N=66)	1.7±0.5 B (N=16)
f) number of pinnae	18.0±3.1 B (N=20)	23.6±6.5 A (N=34)	14.2±4.4 C (N=60)	14.3±2.0 BC (N=15)
g) plant length (cm)	22.3±5.4 B (N=19)	36.2±15.9 A (N=44)	12.2±5.4 C (N=126)	17.8±6.4 B (N=23)
h) ratio of the characteristics (c) to (d) (c/d)	6.2±1.4 B (N=20)	8.0±2.3 A (N=34)	4.7±1.3 C (N=66)	4.7±1.0 C (N=16)
i) ratio of the characteristics (d) to (e) (d/e)	2.8±0.6 C (N=20)	3.2±1.3 C (N=34)	5.9±1.9 A (N=66)	4.8±1.3 B (N=16)

¹ Mean±S.D. (N=sample size), Letters (A–C) indicate significant differences at $p < 0.05$ determined by Tukey-Kramer HSD test.

es within the ingroup was generally weak (48–69%), with the exception of a clade including *Pedicularis verticillata* and *P. lineata* (100%). The Hakusan populations and *P. spicata* were sister to this clade with 48% bootstrap support. In the MP analysis for the *trnK* region, 27 MP trees, requiring 90 steps, were obtained. CI was 0.9222 and RI was 0.8814. The strict consensus tree of the 27 trees had essentially the same topology as the NJ tree, although several weakly supported branches in the NJ tree were unresolved polytomies (Fig. 5).

The topologies of the tree using the ITS region and the *trnK* region were significantly different from each other, particularly in *Pedicularis lineata* (Figs. 4 and 5). The incongruence length difference (ILD; Farris *et al.* 1994) test revealed that the ITS and the *trnK* region data were significantly incongruent ($p = 0.001$).

Morphological analyses

Using herbarium specimens and our field collections, we compared nine morphological characteristics among the Hakusan populations and

the three species of *Pedicularis* (Table 2). The angle of the apex of the galea (a) did not differ significantly between the Hakusan and *P. spicata* samples (mean, 52.9° and 50.8°, respectively). Those values were smaller than for *P. verticillata* and *P. refracta*. The corolla (b) of the Hakusan samples was the shortest (mean, 8.9 mm) among the four samples, while the corolla of *P. refracta* was the longest (mean, 15.1 mm). The length of the leaf blade (c) of the Hakusan populations, *P. spicata*, and *P. refracta* was almost the same (mean, 34.6–43.2 mm), while the leaf blades of *P. verticillata* were the shortest (mean, 20.0 mm). The Hakusan specimens were relatively wider between the rachis and longest pinna (d) (mean, 6.8 mm), but not significantly different from *P. spicata*. The width of the lamina between the rachis and sinus of the pinnae (e) was greatest (mean, 2.5 mm) in samples from Hakusan. The number of pinnae per leaf (f) was highest in *P. spicata* (mean, 23.6) and least in *P. verticillata* (mean, 14.2). In addition, *P. spicata* were the largest (mean, 36.2 cm), while *P. verticillata* was the smallest (mean, 12.2 cm). The ratio of c to d, rep-

representing leaf shape (h), the Hakusan populations and *P. spicata* showed relatively large values (mean, 6.2 and 8.0, respectively), while for i, the ratio of d to e, which represents the degree of dissection of the leaf pinnae, the values for the Hakusan populations and *P. spicata* were relatively small (mean, 2.8 and 3.2, respectively).

To determine the relationship of the Hakusan populations with *Pedicularis spicata*, *P. verticillata* and *P. refracta*, we performed PCA of the seven characteristics (a–g) mentioned above gathered from 104 specimens. A scatter diagram using principal components 1 and 2 is shown in Fig. 6. Component 1 explained 51.0% of the total variation. Leaf blade length (+0.49238 in factor loading) and the width between the rachis and sinus of the pinna (+0.44382) contributed to this component (Table 3). Component 2 explained 19.1% of the total variation, with the width between the rachis and the longest pinna (+0.58291) and corolla length (+0.63099) contributing to this component. The cumulative contribution of the two components was 70.1%. Most specimens of *P. verticillata*, *P. refracta*, and *P. spicata* clustered within the range for each species, although specimens of *P. verticillata* and *P. refracta* were continuously distributed (Fig. 6). Specimens of the Hakusan populations were continuously distributed with those of *P. spicata*. Furthermore, a gap was observed between the Hakusan populations–*P. spicata* cluster and the *P. verticillata*–*P. refracta* cluster. The samples from *P. spicata* from Hokkaido were continuous with the continental populations of *P. spicata*.

Description of the Hakusan populations

Herbs, annual, erect, 10–30 cm tall. Caudex with simple slender root and one to several stems. Stems simple or sparsely branched, with four lines of hairs. Leaves often in whorls of four. Petiole of cauline leaves 0.5–1 cm long, narrowly winged, pubescent. Leaf blade oblong-lanceolate to narrowly lanceolate, 3–5 cm long, 0.5–1 cm wide, pinnately parted or pinnately lobed, apex acute; pinnae acute to cuspidate, with callous teeth, sparsely pilose on both surfaces. Inflorescence terminal, spicate, 3–4 cm long at flowering

time, densely many flowered; proximal bracts leaf-like, distal bracts petiolate, lanceolate or rhomboid, acute, 5–10 mm long, 3–5 mm wide, callously cuspidate-dentate, pilose. Flowers July to August. Calyx suburceolate, ca. 4 mm long, 2 mm wide, pilose, obscurely five-toothed. Corolla pink or purple, ca. 13 mm long; corolla tube ca. 9 mm long; galea, ca. 4 mm long, 1.5 mm wide, apex rounded to truncate; lower lip ca. 5 mm long, 8 mm wide, trilobed, lobes orbiculate, ca. 4 mm long, margin glabrous. Stamens 4; filaments 4, 2 pubescent and 2 glabrous.

Discussion

Attributes of the Hakusan populations of *Pedicularis*

In the molecular phylogenetic analysis using ITS of nuclear ribosomal DNA (nrDNA), the Hakusan populations formed a strongly supported clade with *Pedicularis spicata* (Fig. 4). Although the Hakusan populations have been identified as *P. verticillata* (Hakusan Nature Conservation Center 1995), all samples from Hakusan were positioned in another clade. *P. refracta*, *P. lineata* and *P. szetschuanica* were also positioned in a different clade. For the *trnK* region, the sequences of the Hakusan populations and *P. spicata* were identical (Fig. 5). The results indicate that the Hakusan populations are nested within the *P. spicata* clade and should be considered to be members of that taxon.

As mentioned above, the Hakusan populations were identified as *Pedicularis verticillata* (Hakusan Nature Conservation Center 1995), but there are significant morphological differences between the Hakusan populations and *P. verticillata* in all nine characteristics examined using ANOVA (Table 2). In contrast, no significant differences were detected between the Hakusan populations and *P. spicata* with respect to the following characteristics: (a) angle of the apex of the galea, (b) corolla length, (c) leaf blade length, and (d and i) width between rachis and longest pinna. According to Yamazaki (1993), *P. spicata* is distinguished from *P. verticillata* and *P. refracta* as follows: in the corolla, apex of galea rounded or

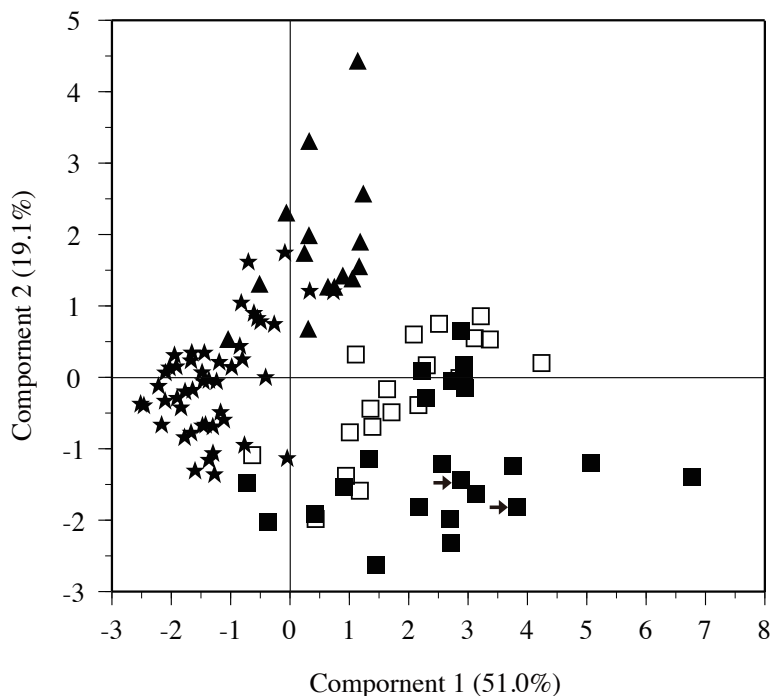


FIG. 6. Scatter diagram showing principal components 1 and 2 for 104 specimens of the Hakusan populations (\square), *Pedicularis spicata* (\blacksquare), *P. verticillata* (\star), and *P. refracta* (\blacktriangle) (see Table 3). The two samples of *P. spicata* with arrow indicate that these localities are at Hokkaido, Japan.

truncate in *Pedicularis spicata*, but truncate in *P. verticillata* and *P. refracta*. The leaves are pinnately lobed or parted in *P. spicata*, while pinnately parted or dissected in *P. verticillata* and *P. refracta*. In our study, no evident differences in the apex of the galea were observed between the Hakusan populations and *P. spicata*. Furthermore, no significant differences between them were observed in the degree of dissection of the leaf pinnae [i: ratio of the characteristics (d) to (e)] (Table 2). In addition, the results of PCA showed overlap in the morphological clusters representing the Hakusan populations and *P. spicata* (Fig. 6), whereas a much clearer gap was evident between the Hakusan populations–*P. spicata* cluster and the clusters representing *P. verticillata* and *P. refracta*. These findings show the Hakusan populations to be morphologically closer to *P. spicata* than to *P. verticillata* or *P. refracta*.

Comparison of the description of the Hakusan populations and descriptions of *Pedicularis spicata* by Tsoong (1963), Yamazaki (1993) and Yang *et al.* (1998) did not reveal distinct morphological differences. Based on the molecular and

morphological evidence, we conclude that the Hakusan populations should be treated as *P. spicata*.

Morphological differentiation of Pedicularis spicata between Hakusan Mtns. and continental eastern Asia

In external morphology, we observed significant differences in several characteristics between the Hakusan plants of *Pedicularis spicata* and *P. spicata* from the Asian mainland (e, f, g, h; Table 2). The leaves of the Hakusan plants are smaller than in *P. spicata* from the mainland. The mainland plants have narrower leaves, but the Hakusan have fewer pinnae, although the length of the leaves was nearly the same in both. These results suggest a possibility of recognizing the Hakusan populations as an infraspecific taxon of *P. spicata*. Two subspecies of *P. spicata* were described by Tsoong (1963): subsp. *bracteata* and *stenocarpa*. The former is distinguished from subsp. *spicata* by its strongly enlarged bracts (10 mm long \times 8 mm wide), while the latter is distinguished by the elongated capsules (8–10 mm

TABLE 3. Factor loading, eigenvalue, contribution, and accumulated contribution of principal components.

Character	Components	
	PC1	PC2
a) angle of apex of the galea	-0.382	0.177
b) corolla length	-0.05150	0.631
c) leaf blade length	0.492	0.095
d) width between rachis and longest pinnae	0.339	0.583
e) width between rachis and sinus of the pinnae	0.444	0.194
f) number of pinnae	0.374	-0.343
g) plant length	0.397	-0.258
Eigenvalue	3.568	1.334
Contribution (%)	50.976	19.050
Accumulated contribution (%)	50.976	70.976

long) (Yang *et al.* 1998). Both the subspecies occur in western Hebei, China (Tsoong 1963, Yang *et al.* 1998). The bracts are not enlarged in the specimens from the Hakusan populations (5–10 mm long, 3–5 mm wide). However, we did not measure the attributes of the capsule. For the capsule, field research must be performed when the plants are in fruit. Thus, we were unable to definitively establish the intraspecific status of the Hakusan populations of *P. spicata* in the present study, and this needs to be addressed in a future study.

Perspectives on disjunct distribution of Pedicularis spicata

Pedicularis spicata occurs mainly in north-eastern Asia (Russia, Mongolia, China, and North Korea) (Fig. 2). In Japan, *P. spicata* has been reported only from the Tokachi district of Hokkaido. Yokoyama (1951) and Takita (2001) postulated that it had been introduced from north-eastern China, and it is treated as naturalized in several reports (Sugimoto 1978, Igarashi 2001). The evidence for its introduced status is based on only a few collections obtained from around a warhorse supplementary place and on roadsides in the vicinity of Ashoro and Nukabira, Tokachi district, Hokkaido [August 16, 1936, *H. Yokoyama* 3222 (TI, SAPS); September 17, 1983, *K. Takita* 5877; and August 21, 1994, *K. Takita* 5993 (SAPT)]. We included these specimens in our morphological analyses, and most characteristics

were within the range of variation of continental specimens (Fig. 6), but the evidence is inconclusive to determine its introduced status on Hokkaido.

This is the first report of the natural occurrence of *Pedicularis spicata* in central Honshu, Japan, revealing a disjunct distribution pattern between the Asian mainland and Honshu Island. The studies of the biogeographic importance of the alpine region of central Honshu by Fujii & Senni (2006) and Ikeda *et al.* (2008) inferred that the alpine region of central Honshu acted as a refugium for alpine plants during the interglacial periods of the Pleistocene. Our study suggests that this may be the case for *P. spicata*. Based on the assumption that *P. spicata* originated on the Asian mainland, it is likely that the Hakusan populations represent surviving remnants of plants that reached Japan during cooler glacial periods.

It is unclear why *Pedicularis spicata* occurs solely on the Hakusan population, while other high mountains in central Honshu also appears suitable. While many alpine plants occur on Hakusan Mtns., none are endemic (Shimizu 1992, the Hakusan Nature Conservation Center 1995), with the exception of *Cirsium occidentalinipponense* Kadota on Mt. Sannomine in the southern part of the Hakusan Mtns. (Kadota 1997) and some taxa at the rank of form (Satomi & Hashimoto 1970). The main alpine areas of Hakusan Mtns. are composed of volcanic rocks from eruptions that have taken place from approximately 150,000 years ago to the present. The Hakusan populations of *P. spicata*, however, occur on relatively old volcanic rocks or on old sedimentary rocks of the Jurassic–Cretaceous Tetori Group (Geological Survey of Japan 2012). The Hakusan populations are found on gravel exposures, a habitat that other plant species are unable to occupy easily. These factors may reflect the relict occurrence of the Hakusan populations of *P. spicata*, although they are insufficient for explaining why the species is absent elsewhere in Japan.

We thank Susumu Okitsu, Tadashi Kariyazaki, Sumio Sei, and Hiroshi Tsuji for their cooperation in collecting

the samples, Takayuki Azuma, Jin Murata, and Tetsuo Ohi-Toma for their cooperation in studying specimens. We also thank the directors and curators of KANA, SAPS, SAPT and TI for granting permission to examine specimens. This study was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (No. 20241056 to H. Takahashi).

References

- Doyle, J. J. & J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11–15.
- Farris, J. S., M. Källersjö, A. G. Kluge & C. Bult. 1994. Testing significance of incongruence. *Cladistics* 10: 315–319.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- Fujii, N., K. Ueda & T. Shimizu. 1996. Intraspecific sequence variation of chloroplast DNA in Japanese alpine plants. *J. Phytogeogr. Taxon.* 44: 72–81.
- Fujii, N. & K. Senni. 2006. Phylogeography of Japanese alpine plants: biogeographic importance of alpine region of central Honshu in Japan. *Taxon* 55: 43–52.
- Fujii, N., K. Ueda, Y. Watano & T. Shimizu. 2013. Taxonomic revival of *Pedicularis japonica* from *P. chamissonis* (Orobanchaceae). *Acta Phytotax. Geobot.* 63: 87–97.
- Geological Survey of Japan, AIST (ed.). 2012. Seamless digital geological map of Japan 1: 200,000. Jul 3, 2012 version. Research Information Database DB084, Geological Survey of Japan, National Institute of Advanced Industrial Science and Technology, Tsukuba.
- Grubov, V. I. 2001. Key to the Vascular Plants of Mongolia (with an atlas) vol. 2. Science Publishers, Inc., Enfield.
- Hakusan Nature Conservation Center 1995. The report of inventory research for higher plants in Hakusan Mountains. Ishikawa Prefecture. Ishikawa (in Japanese).
- Hashimoto, M. & N. Satomi. 1969. Hakusan Mountains from the point of view of plant taxonomy and phyto-geography. *In: Ishikawa Prefecture & Nature Conservation Society of Japan (eds.). The interim report of Hakusan resource survey project, vol. 3. pp. 7–45. Kanazawa (in Japanese).*
- Igarashi, H. 2001. The Handbook of Naturalized Plants in Hokkaido. Hokkaido Research Institute for Wild Plants, Sapporo (in Japanese).
- Ikeda, H., K. Senni, N. Fujii & H. Setoguchi. 2008. Survival and genetic divergence of an arctic-alpine plant, *Diapensia lapponica* subsp. *obovata* (Fr. Schm.) Hultén (Diapensiaceae), in the high mountains of central Japan during climatic oscillations. *Pl. Syst. Evol.* 272: 197–210.
- Ivanina, L. I. 1991. *Pedicularis* L. *In: Charkevich, S. S. (ed.), Plantae Vasculares Orientis Extremi Sovietici, vol. 5. pp. 334–359. Nauka, Saint Petersburg (in Russian).*
- Iwatsuki, K., T. Yamazaki, D. E. Boufford & H. Ohba (eds.). 1993. Flora of Japan, Vol. IIIa. Kodansha, Tokyo.
- Iwatsuki, K., T. Yamazaki, D. E. Boufford & H. Ohba (eds.). 1995a. Flora of Japan, Vol. IIIb. Kodansha, Tokyo.
- Iwatsuki, K., T. Yamazaki, D. E. Boufford & H. Ohba (eds.). 1995b. Flora of Japan, Vol. I Kodansha, Tokyo.
- Iwatsuki, K., D. E. Boufford & H. Ohba (eds.). 1999. Flora of Japan, Vol. IIc. Kodansha, Tokyo.
- Iwatsuki, K., D. E. Boufford & H. Ohba (eds.). 2001. Flora of Japan, Vol. IIb. Kodansha, Tokyo.
- Iwatsuki, K., D. E. Boufford & H. Ohba (eds.). 2006. Flora of Japan, Vol. IIa. Kodansha, Tokyo.
- Kadota, Y. 1997. Taxonomic studies of *Cirsium* (Asteraceae) in Japan: 3. *Cirsium occidentalinipponense*, sp. nov. with special reference to the lectotypification of *Cirsium borealinipponense* Kitam. *Bull. Natl. Sci. Mus. Ser. B Bot.* 23: 115–125.
- Kadota, Y. 2012. Systematic studies of Asian *Aconitum* (Ranunculaceae) XIV. Four new species of subgenus *Lycotconum* from Hokkaido, northern Japan. *J. Jap. Bot.* 82: 1–21.
- Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111–120.
- Murata, J. 2000. Flora of Japan. *In: Iwatsuki K. & M. Kato (eds.). Floristic research (the botany of biodiversity 1). University of Tokyo Press, Tokyo (in Japanese).*
- Oda, J. & H. Nagamasu. 2011. *Carex noguchii* (sect. *Rarae*, Cyperaceae), a new species from Japan. *Acta Phytotax. Geobot.* 61: 145–150.
- Ohwi, J. 1965. Flora of Japan (English ed.). Smithsonian Institution, Washington.
- Ree, R. H. 2005. Phylogeny and the evolution of floral diversity in *Pedicularis* (Orobanchaceae). *Int. J. Pl. Sci.* 166: 595–613.
- Satomi, N. 1983. Flora of Ishikawa Prefecture. Ishikawa Prefecture, Kanazawa (in Japanese).
- Satomi, N. & M. Hashimoto. 1970. New plants and first discovery plants in the Hakusan Mtns. *In: Nature Conservation Society of Japan (Hakusan academic investigation team, Chubu Branch) (ed.). Nature of Hakusan (Hakusan no shizen), pp. 217–223. Ishikawa Prefecture, Kanazawa (in Japanese).*
- Setoguchi, H. & Y. Maeda. 2010. A new species of *Pieris* (Ericaceae) from Amamiyoshima, Ryukyu Islands, Japan. *Acta Phytotax. Geobot.* 60: 159–162.
- Shimizu, T. 1992. Preface to the plants of Hakusan Moun-

- tains (Hakusan no syokubutsu ni yosete). Hakusan (a public magazine of Hakusan Nature Center, Ishikawa Prefecture) 19 (4): 6–13 (in Japanese).
- Shimizu, T. 1997. Flora of Nagano Prefecture. Shinano Mainichi Shinbunsha, Nagano (in Japanese).
- Suetsugu, K., M. Nakama, T. Watanabe, H. Watanabe & M. Yokota. 2012. The northernmost locality of *Gastrodia shimizuana* (Orchidaceae). *J. Jap. Bot.* 87: 62–64.
- Sugawara, T. 2012. A taxonomic study of *Asarum celsum* and its allies (Aristolochiaceae) on Amami-oshima, Southwestern Kyushu, Japan. *Acta Phytotax. Geobot.* 62: 61–68.
- Sugimoto, J. 1978. New keys to herbaceous plants of Japan I, Dicotyledoneae, revised and enlarged edition, Inoue Book Company, Tokyo (in Japanese).
- Swofford, D. L. 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10, Sinauer Associates, Sunderland.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei & S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.
- Takita, K. 2001. Illustrations of Hokkaido flora (Hokkaido syokubutsu zufu). Kushiro (in Japanese).
- Tsoong, P. C. 1963. Scrophulariaceae (Para II). *In*: Chien, S. S. & W. Y. Chun. (eds.) *Fl. Reipubl. Popularis Sin.* vol. 68, pp. 1–378. Science Press, Beijing (in Chinese).
- White, T. J., T. Bruns, S. Lee & J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M., D. Gelfand, J. Sninsky & T. White. (eds.) *PCR protocols: a guide to methods and applications*, pp. 315–322. Academic Press, San Diego.
- Yamazaki, T. 1993. *Pedicularis*. *In*: Iwatsuki, K., T. Yamazaki, D. E. Boufford & H. Ohba. (eds.) *Flora of Japan IIIa*. pp. 364–371. Kodansha, Tokyo.
- Yang, F. S. & X. Q. Wang. 2007. Extensive length variation in the cpDNA *trnT-trnF* region of hemiparasitic *Pedicularis* and its phylogenetic implications. *Pl. Syst. Evol.* 264: 251–264.
- Yang, H., N. H. Holmgren & R. R. Mill. 1998. *Pedicularis*. *In*: Wu, Z. Y. & P. H. Raven. (eds.) *Flora of China*, vol. 18, pp. 97–209. Science Press, Beijing & Missouri Botanical Garden Press, St. Louis.
- Young, N. D., K. E. Steiner & C. W. Depamphilis. 1999. The evolution of parasitism in Scrophulariaceae/Orobanchaceae: plastid gene sequences refute an evolutionary transition series. *Ann. Missouri Bot. Gard.* 86: 876–893.
- Yokoyama, H. 1951. Flora of Tokachi District (Tokachi Syokubutsushi). Obihiro regional forestry office, Obihiro (in Japanese).

Received April 25, 2013; accepted March 4, 2014